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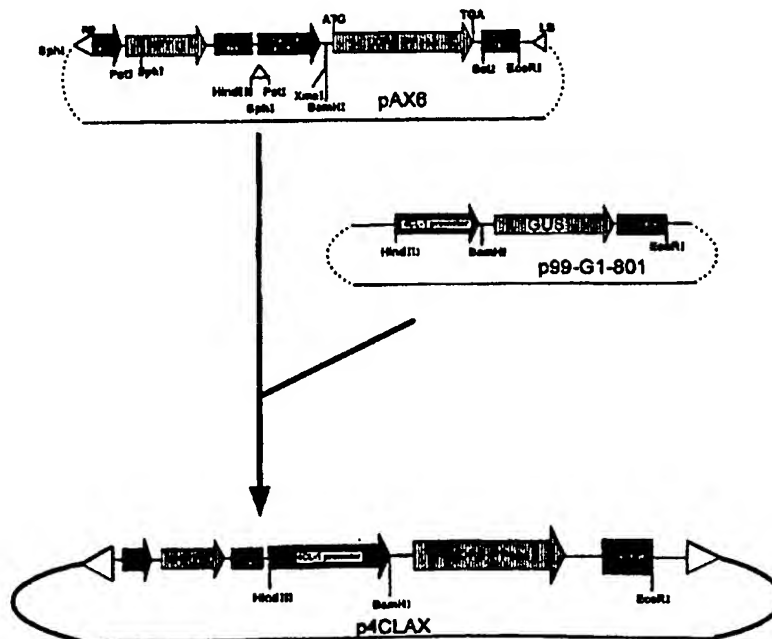
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(57) Abstract

A process of increasing plant growth and yield and increasing resistance to stress comprises introducing into the plant a DNA sequence encoding a product which modifies, in the plant, the level of cellulose precursors.

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TITLE: A PROCESS OF INCREASING PLANT GROWTH AND YIELD AND MODIFYING CELLULOSE PRODUCTION IN PLANTS

FIELD OF THE INVENTION

The present invention relates to processes of enhancing plant growth and productivity and more specifically, to the field of carbon re-allocation in plants.

BACKGROUND OF THE INVENTION

Increasing harvestable plant yield is a major goal of all plant breeding efforts. In fibre producing crops, the economic value of this yield is directly related to the amount, location, and length of the cellulose fibres. It has been suggested that cellulose content and fibre yield is limited by the amount of substrate, or sugars, produced during photosynthesis. However, numerous studies provide evidence that although crucial for plant growth and survival, the availability of carbohydrates derived from photosynthesis are not major limiting factors in cellulose synthesis. Thus there exists a substantial opportunity to increase fibre yield by creating a sink for this existing photosynthate in cells high in cellulose. Sucrose, the major form of translocatable carbohydrate produced during photosynthesis in the plant, is translocated to sink tissue where it is converted to other compounds such as starch or cellulose.

Despite the fact that the amount of photosynthates in the plant are not a primary limitation in cellulose content, the rate of photosynthesis plays a large role in the overall growth of a plant. Further, one element in the control of photosynthesis in the plant is the feedback-inhibition of photosynthesis by photosynthetic products, such as starch, sucrose and hexose sugars. Goldschmidt and Huber (1992)¹ tested the effect of girdling the leaves of crop plants and demonstrated that the build up of starch and other products of photosynthesis actually inhibited the rate of photosynthesis. These findings, and others (Sonnewald & Willmitzer 1992)², indicate the photosynthetic rate, and ultimately plant growth, may be directly correlated with the rate that photosynthates are drawn away from the leaf, or the rate of biosynthetic degradation in the leaves. The degradation of photosynthates occurs primarily in cells/tissues that are actively growing (meristematic or young tissues) or in tissues where photosynthates are utilized for storage or structural components (sink tissues). Therefore altering the rate that carbohydrates are translocated to these sink tissues (altering carbon allocation) would not only increase overall plant growth (remove inhibitors of photosynthesis), but also increase the amount of storage (starch) or structural components (cellulose).

A striking example of the benefits of altering carbon allocation has been demonstrated in potato. By increasing the synthesis and accumulation of ADP-glucose in the tuber, starch synthesis increased which significantly increased dry matter content. In fact, this resulted in a 25% increase in tuber yield. The increase in ADP-glucose in the tuber was accomplished by genetically engineering the potato with a

bacterial ADP-glucose pyrophosphorylase gene controlled by a tuber specific promoter (Shewmaker and Stalker 1992)³.

Much like ADP-glucose is a precursor to starch synthesis, the nucleotide sugar UDP-Glucose, (UDPG), is a high energy substrate for cellulose biosynthesis in both bacteria and higher plants (Delmer 1987⁴, Delmer et al. 1995⁵). Several bacterial genes which encode the enzyme UDP-glucose pyrophosphorylase (UDPG-PPase), responsible for the synthesis of UDPG, have been isolated (Ross et al. 1991⁶). An existing patent by Betlach (1987⁷) claims increased synthesis of xanthan and other polysaccharides in bacteria by insertion of a UDPG-PPase gene from *Xanthomonas campestris*. However, the claims in this patent are limited to increasing polysaccharide biosynthesis in prokaryotic organisms.

It is an object of the present invention to obviate or mitigate the above disadvantages.

SUMMARY OF THE INVENTION

The present invention provides a process of increasing plant growth and yield and increasing plant resistance to stress which comprises introducing into a plant a DNA sequence encoding a product which modifies, in the plant, the level of cellulose precursors. This product of the present invention includes, but is not limited to, ribonucleic acid ("RNA") molecules, enzymes related to cellulose biosynthesis and proteins which regulate the expression of these enzymes.

It has been found that the process of the present invention leads to the reallocation by simple diffusion of carbohydrates such as glucose from photosynthetic cells, such as the leaf cells, to other cells within the plant. This translocation removes the inhibition on photosynthesis imposed by excess photosynthate accumulation in these photosynthetic cells thereby allowing the plant to produce more simple sugars by continued photosynthesis. In other words, as photosynthesis continues in an uninhibited fashion, more simple sugars are produced than would have otherwise have been possible. These simple sugars are building blocks for plant growth via the production of polymers such as starch and cellulose.

Further, the present invention provides a process of modifying the production of cellulose in a plant which comprises introducing into said plant a DNA sequence encoding a product which modifies, in the plant, the level of cellulose substrates. As above, the product includes, but is not limited to, ribonucleic acid ("RNA") molecules, enzymes related to cellulose biosynthesis and proteins which regulate the expression of these enzymes.

The subject invention also provides a plant having increased growth and yield, increased resistance to stress and/or modified cellulose producing activity as a result of introducing into said plant or parent of said plant a DNA sequence coding for a product which modifies the level of cellulose precursors in the plant.

Another aspect of the present invention provides for a DNA expression vector comprising a DNA sequence encoding a product which modifies, in a host, the level of cellulose precursors, said sequence being operably linked to an expression effecting DNA sequence and flanked by translational start and stop sequences.

The present invention also provides a genetically modified seed comprising a DNA sequence, said sequence encoding a product capable of increasing growth and yield and/or modifying the level of cellulose precursors in the plant resulting from said seed.

There are two primary features of the process of the present invention. Firstly, in all plants regardless of whether they are fibre-producing (trees, hemp, cotton etc..) or not, what is achieved are plants having faster rates of growth and increased yield by non-specifically re-allocating carbon within the plant away from photosynthetic cells. This allows photosynthesis to continue uninhibited to produce more simple "construction" sugars thereby enhancing the efficiency of the plant growth rate and increasing growth yield. Secondly, in fibre-producing plants, the expression of the DNA sequence introduced into the plant may be targeted to specific individual cell types within the plant to increase predictably cellulose deposition in a cell specific manner. In forest trees, this is expected to increase wood production and fiber yield, especially when the gene is linked to a promoter which expresses only in wood forming tissues. Increased fiber yield can also be expected in other non-forestry fiber producing plants, such as hemp and sisal. In addition to targeting wood forming tissues, increased cellulose production can be obtained in other parts of the plant such as the bolls surrounding the seeds of cotton plants.

Specific applications for increased cellulose synthesis include numerous crops with diverse uses and growth habits. In forestry, wood production is influenced by a combination of physiological and biochemical processes governed by substantial genetic variation. This has lead to the theoretical consideration of limitations on increasing yield due to fundamental constraints on energy supply (Farnum 1983¹). Despite such limitations, increases in tree growth of 50 to 300% are possible depending on the tree species and growing environment. Clearly, improving energy capture, conversion of radiant energy, and altering carbon allocation within the plant are promising areas for tree improvement. Increasing cellulose content by the processes outlined herein can achieve such gains.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is described by way of the following non-limiting drawings in which:

Figure 1 illustrates schematically the formation gene cassette comprising the UDPG-PPase gene and CaMV promoter, the cloning vector pUC comprising the gene cassette and transformation vectors pBI121 and pAX6 comprising the gene cassette and preferential promoters;

Figure 2 represents the nucleotide sequence of the cloned gene cassettes in the pBI series of binary vectors;

Figure 3 illustrates schematically the formation of the xylem specific transformation vector with the UDPG-PPase gene;

Figure 4 represents an assay of UDPG-PPase activity in tobacco plants;

Figure 5 is a graph representing the titre of anti-UDPG-PPase sera with affinity purification;

Figure 6 is a Western Blot analysis of UDPG-PPase protein with the anti-UDPG-PPase antibody;

Figure 7 is a bar graph representing an analysis of cellulose in transformed tobacco plants;

Figure 8 is a photographic representation of plants of CaMV 35S-UDPG-PPase transgenic and non-transformed control tobacco plants under nutrient and water stress:

Figure 9 is a bar graph representing the height of CaMV 35S-UDPG-PPase transgenic (T33-7, T37-2, T42-4, T44-2) and non-transformed (C8-5, C8-6) control tobacco plants with the first 40 days under water and nutrient stress;

Figure 10 is a bar graph showing total dry weight (stems, leaves and roots) of CaMV 35S-UDPG-PPase transgenic (T33-7, T37-2, T42-4, T44-2) and non-transformed (C8-5, C8-6) control tobacco plants with the first 40 days under water and nutrient stress;

Figure 11 is a bar graph the harvest 3 (final harvest) broken down into the three major plant parts from CaMV 35S-UDPG-PPase transgenic (T33-7, T37-2, T42-4, T44-2) and non-transformed (C8-5, C8-6) control tobacco plants with the first 40 days under water and nutrient stress;

Figure 12 is a line graph showing UDPG-PPase enzyme activity in the stems at the final harvest (80 days) in stressed controls and 35S-UDPG-PPase transgenic tobacco;

Figure 13 is a bar graph showing the height of control and 4CL-UDPG-PPase Tm tobacco plants at three intervals during growth; and

Figure 14 is a comparison of bar graphs showing the distribution of biomass in various parts of the plant in control and 4CL-UDPG-PPase Tm transformed tobacco plants at maturity.

PREFERRED EMBODIMENTS OF THE INVENTION

The present invention affords the ability to increase plant growth rates and yield and increased resistance to stress through the addition to the plants of DNA sequences encoding products which have a modifying role on the level of cellulose precursors. The result of the introduction and expression in the plant of this DNA sequence is the beneficial and optionally selective allocation of carbon within the plant.

In a preferred form of the invention described further hereinbelow, the DNA sequence may be selectively expressed in cells primarily responsible for cellulose synthesis. By creating a sink for these carbohydrates in cellulose producing cells, excess photosynthate can be diverted to these cells where the default pathway for their use would be conversion into cellulose, thereby increasing cellulose content in the plant. For example, in trees, as photosynthate such as sucrose and hexose sugars are removed from the leaves to the stem the inhibitory effect of these compounds on photosynthesis is removed. This has huge implications in forestry, because whether harvesting for lumber or fiber, the product is cellulose. The benefit of increased cellulose is not limited, however, to forestry, as there are numerous other fiber crops including sisal, cotton, and hemp.

In addition, what has been found to occur in plants engineered to express or overexpress DNA sequences encoding products which have a modifying role on the level of cellulose precursors is that there is an increased resistance to stress as compared to the control plants. Stress includes, but is limited to water or osmotic stress, nutrient stress, environmental stresses such as temperature and pH as well as chemical stress. Furthermore, it has been found that the plants engineered in accordance with the present invention (or progeny thereof) have sustained or even increased growth under conditions of stress. This was simply not the case with control plants.

In one embodiment of the present invention, the product encoded by the inserted DNA sequence is an enzyme such as a carbohydrate-modifying enzyme selected from the group consisting of uridine diphosphate-glucose pyrophosphorylase ("UDPG-PPase"), sucrose synthetase, cellulose synthase or any derivative thereof. Sucrose synthetase is responsible for the synthesis of uridine diphosphate-glucose ("UDP-glucose") in plants. The present application is not limited to the specific enzymes disclosed herein as these are intended merely as a sampling of preferred enzymes. What is required for the enzymes to be useful herein is that they have the potential to effect, in some way, the level of cellulose precursors in the plant. These enzymes may originate from any organism including other plant species, bacteria or yeast.

Although bacterial enzymes are preferred for the reasons described below, it is to be understood that the DNA sequences encoding enzymes may originate from many other organisms. The key criteria in selecting a "preferred" enzyme is a relatively high K_m value for the product as compared to the precursors or substrates thereby indicating a preference in the reaction toward the product.

UDP-PPase is the most preferred enzyme particularly when the DNA sequence encoding the enzyme originates from bacteria. The enzyme kinetics data (UDPG-PPase has a relatively high K_m value for UDPG as compared to the substrates UTP, glucose-1-phosphate and PPi), lack of signal sequences and the fact that, unlike the corresponding plant gene, the bacterial UDPG-PPase gene is not strongly inhibited by UDPG accumulation make the bacterial UDPG-PPase gene an excellent target gene to increase UDPG levels in plants. Additionally, bacterial genes are widely available and are less likely to lead to co-suppression of the native UDPG-PPase genes. Bacterial genes may be selected from many commonly available genera, but in a preferred form are selected from the genus *Acetobacter*, more specifically from the species including *Acetobacter xylinum* and from the genus *Xanthomonas*.

In an alternative embodiment, the DNA sequence introduced into the plant may encode regulatory, feedback or other proteins which effect cellulose biosynthesis in plants or any derivatives thereof. These include lignin-modifying proteins and proteins which regulate lignin-modifying proteins.

In a further embodiment, the DNA sequence introduced into the plant encodes for an RNA molecule having regulatory properties. For example, these RNA molecules may effect enzyme synthesis, cellulose synthesis or may indirectly modify cellulose synthesis through an alteration in precursor or lignin synthesis.

Prior to the introduction of the DNA sequence into the plant cells as described further hereinbelow, the DNA sequence or gene of interest (terms "DNA sequence" and "gene" used hereinafter interchangeably) encoding for the product with cellulose modulatory effects is prepared into a DNA construct or vector. Initially, the gene of interest is extracted by known techniques from the source (for example, bacteria, yeast or other plant species..) or obtained from a depository such as ATCC. The general extraction procedure involves lysing the cells of the source and recovering the released DNA through extraction such as phenol/chloroform with a final precipitation in, for example, alcohol.

The gene or DNA sequence is then amplified by, for example, the polymerase chain reaction ("PCR") and subsequently cloned into the desired construct or vector. The amplification of the gene based on the PCR makes use of primers and inducing agents, sometimes referred to as enzyme catalysts. The PCR process is described in considerable detail in US Patent No. 4,800,159 and Canadian Patent No. 1,237,685 both to Cetus Corporation and in US Patent Nos. 4,965,188 and 4,682,202 all of which are incorporated herein by reference.

The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of DNA synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to the nucleotide sequence is induced, i.e. in the presence of nucleotides and inducing agent and at a suitable pH and temperature. The primer is preferable single-stranded for maximum efficiency in amplification but may alternatively be double-stranded. If double-stranded, the primer is first treated to separate its strands before being used to prepare the extension products. Preferably, the primer is an

oligodeoxyribonucleotide. The exact lengths of the primers may be different for each DNA sequence or "template" to be amplified. Generally, a balance must be struck with respect to primer size. It must be large enough to be usefully specific to the template, that is, it must be homologous to a large enough region of the template so that other extraneous DNA (not related to the DNA sequence) with some degree of homology to the primer is not amplified to a significant extent. On the other hand, the size of the primer should not be so large as to be unwieldy and prohibitive in terms of time and cost. This balance may be achieved for most of the DNA sequences contemplated within the scope of the present invention with primer of between 10-50 nucleotides in length. The determination of the appropriate lengths of primers, however, is well within the purview of a technician of average skill in this area. In addition, although the PCR is an efficient process for producing exponential quantities of a DNA product relative to the number of reaction steps involved, other known DNA amplification techniques may be used within the scope of the present invention.

Suitable constructs or vectors for transforming the plant host are well known in the art and include plasmids, cosmids, phage derivatives, phasmids and expression vectors. General vectors of interest may contain an origin of replication functional in one or more plant species, convenient restriction endonuclease digestion sites and selectable markers for the plant cell. Preferred transformation vectors vary depending on the particular host but include Bluescript vectors, pBI (*Agrobacterium* binary vectors) and pUC derived vectors. Other vectors useful for assessing mRNA and protein expression in plants include pMAL and pGEM vectors.

In order to achieve expression of the DNA sequence of interest in a plant host, it may be necessary to make modifications to the regulatory and/or controlling sequences of that DNA. Specifically, it may be necessary to link the gene of interest operably to an expression effecting DNA sequence, such as one or more promoters and to flank it with translational start and stop signals. In particular, the start codon may be changed and suitable plant promoter and terminator sequences added. Optionally, an improved translation consensus sequences may be provided. It is to be understood, however, that these modifications need not be made for each and every DNA sequence contemplated within the scope of the present invention. The question of making these technical modifications is well within the purview of a technician of average skill in this field.

A number of promoters may be ligated to the DNA sequence, the most efficient type of which varies between plant hosts. In a preferred form, the promoter expresses specifically in vascular plant cells or cellulose-producing cells within the plant. For example, in trees, xylem specific promoters including, but not limited to the 4-coumarate CoA ligase ("4CL") promoter from parsley are preferred in order to direct expression to wood-forming tissues. In tobacco plants, suitable promoters include the cauliflower mosaic virus ("CaMV") 35S promoter. In other plant species, 4CL and CaMV 35S among others may be used.

For consistency in terminology, the DNA sequence to be transformed having modifications to the regulatory and/or controlling sequences is referred to hereinafter as a "gene cassette" or "DNA sequence cassette". Transformation of this DNA sequence cassette into a plant host may be achieved by a number of established methods. Generally for most plants including tobacco, the widely practised *Agrobacterium* transformation method is appropriate. General techniques for transformation of plants can be found in Svab Z.P. Hajdukiewicz and P. Maliga. 1995. Generation of Transgenic Tobacco Plants by *Agrobacterium* Transformation. pp. 61-77. (eds. P. Maliga, D.F. Klessig, A.R. Cashmore, W. Gruissem and J.E. Vamer) *Methods in Plant Molecular Biology*, Cold Spring Harbor Laboratory Press, New York and Horsch. R.B., J.E. Fry, N.L. Hoffman, D. Eichholtz, S.G. Rogers and R.T. Fraley. 1985. A Simple and General Method for Transferring Genes into Plants. *Science* 227:1229-1231 both of which are incorporated herein by reference. In a preferred form for trees, in particular coniferous species such as spruce, the particle gun bombardment method may be used in conjunction with embryonic cultures.

In the particle gun bombardment process, which is described in more detail in the incorporated reference: Ellis et al. 1993. Stable Transformation of *Picea glauca* by Particle Acceleration. *Bio/Technology*. vol. 11 pp. 84-89, embryonic cultures of the plant host are exposed, for short time, to a blast or bombardment of the DNA sequence or DNA sequence cassette to be transformed. Generally, this is achieved by inert gas (such as helium) propulsion of micro-particles of gold coated with the DNA sequence to be transformed. Optionally, the DNA sequence may be fused to a marker gene, such as an antibiotic resistance gene to allow for subsequent selection of cultures for further regeneration. For example, the DNA sequence may be fused to a kanamycin resistance gene and the transformed cultures thereafter selected for plant regeneration on the basis of kanamycin resistance.

After transformation, the plant tissue is preferably placed on an antibiotic containing medium on which the transformed cells expressing a resistance gene are able to grow. Non-transformed cells are thereby retarded in their growth and/or die on the antibiotic. In this manner, once plants are regenerated either through the formation of shoots or the development of mature embryos and germination (as is the case with somatic embryogenesis), only plants capable of expressing the introduced genes (the DNA sequence of the present invention together with the antibiotic resistance gene) are produced.

The seeds (including artificial seeds derived from somatic embryos) and subsequent plants resulting from the transformation and regeneration process as described herein have increased rates of growth, increased yields and increased resistance to stress as a result of the transformed DNA sequence which modifies the level of cellulose precursors in the plant. For example, if the transformed DNA sequence comprises the UDPG-PPase gene, glucose (a photosynthate) is converted in plant cells to UDP-glucose (a high energy substrate for cellulose biosynthesis). As these plant cells then have a lesser concentration of glucose relative to photosynthetic cells, glucose translocates by simple diffusion to these cells. This carbon translocation reduces the inhibition of excess photosynthate on photosynthesis leading to more efficient photosynthesis

and enhanced sugar production. In a preferred form, when the DNA sequence expression is targeted to vascular or cellulose-producing cells via specific promoters, not only is there carbon re-allocation as described above, but there is provided more UDP-glucose in these cells allowing for enhanced cellulose production with the attendant advantages.

EXAMPLES

Summary

Transformation of tobacco with a construct for overexpression of an *Acetobacter xylinum* (Ax) UDPG-PPase gene has resulted in increased dry weight, solute content, and a-cellulose content in the transformants relative to non-transformed control plants. Antibody specific to the Ax UDPG-PPase gene has been generated in rabbits following injection of a fusion protein overproduced in *E. coli*. Using this antibody, detection of expressed UDPG-PPase in transgenic tobacco has been confirmed. The inserted gene cassette segregated based on kanamycin resistance in most of the T₁ population in a manner consistent with a single insertion site. Initial experiments with the transformation of two constructs, a 4CL-GUS and a 4CL-UDPG-PPase in spruce has yielded numerous putative transformed lines. These lines are currently undergoing GUS screening (4CL-GUS) and further kanamycin screening (both constructs).

Example 1: Preparation of DNA constructs

The original bacterium (*Acetobacter xylinum*) containing the gene UDP-glucose pyrophosphorylase (UDPG-PPase) was obtained from ATCC (23768). The UDPG-PPase gene was amplified by PCR and subsequently cloned. Design of the PCR primers included the following considerations:

- Addition of restriction sites suitable for cloning into a variety of transformation vectors.
- Mutation of the start codon from valine to methionine
- Mutation of internal Eco RI site to remove it without change in amino acid sequence.
- Addition of non-coding DNA fragment at the 5' end of the gene to enhance the efficiency of translation.

The resulting primer A at the 5' end is:

M A K P L K K A V L P

taGGATCCgtcgaccATGGTCAAccccttaaaaagccgtattgc

and the original UDPG-PPase gene at 5' end is:

ttgaggtaaatattaGTGATTAAgccccttaaaaagccgtattgccggttg-->

V I K P L K K A V L P

The original UDPG-PPase gene at the 3' end is:

ggtgccggaagatcacttgtacttcgtcaggaattcacgcacgccggg

Stop codc * S N V C A P

and the primer B at the 3' end is:

ggtgccTCTAGAtcACTTGTacttcgtcagGACTTCcacgcacgccggg

Stop code * S N* V C A P

Amplification of the gene was successful and DNA sequencing confirmed that the amplified fragment was the UDPG-PPase gene. The amplified UDPG-PPase gene from *A. xylinum* is almost identical to the published sequences.

The complete DNA sequence has been analyzed to detect potential exon and intron splice sites. Several characters have been considered to find introns which could potentially cause splicing of the mRNA. These include no-random codons in the DNA sequence, preferential usage of certain codons, GC content, relative positions of purines and pyrimidines in the codons of known sequences, and exon value of regions between splice sites, as well as downstream and upstream of the end of the exon. Analysis showed that the potential of mRNA splicing in plant tissue was low.

The amplified UDPG-PPase sequence was cloned into a BlueScript vector and used for construction of the gene cassettes. The gene cassette was constructed by ligating the UDPG-PPase gene with a CaMV 35S promoter. A pUC based cloning vector containing the UDPG-PPase gene with suitable restriction sites for

placing the "gene cassette" in a variety of transformation vectors containing xylem preferential and other promoters was made. Fig. 1 shows that gene cassettes and vectors.

Several vectors containing the UDPG-PPase gene from *Acetobacter xylinum* (Ax) have been derived from the gene cassette for different purposes, as follows:

For tobacco transformation:

- pBI₁Ax - *Agrobacterium* binary vector with the Ax gene linked to CaMV 35S promoter
- pBI₄CLAx - *Agrobacterium* binary vector with the Ax gene linked to a parsley 4CL promoter for xylem preferential expression

For spruce transformation:

- pBI₄CLAX - pUC-derived vector, containing Ax linked to 4CL promoter
- p₄CLGUS - pUC-derived vector containing GUS linked to a parsley 4CL promoter for assessment of xylem specificity of the promoter

For assessing mRNA and protein expression in transformants:

- pMAL - protein expression vector in *E. coli*, for antibody production.
- pGem - mRNA transcriptional vector, for *in situ* hybridization.

pBI based binary vectors have been constructed by ligation of UDPG-PPase gene into pBI121 for *Agrobacterium* transformation. The resultant binary vector contains a transcriptional fusion of the UDPG-PPase gene to the CaMV 35S promoter. The identity of the cloned gene cassettes in the pBI series of binary vector was confirmed by DNA sequencing (Fig. 2). The 4-coumarate CoA ligase (4CL) promoter from parsley has been identified as a xylem preferential promoter and the 4CL promoter is highly specific for xylem expression in transgenic tobacco. The 4CL promoter was modified and ligated to the UDPG-PPase gene. The construct was subsequently placed in a binary vector containing the 4CL promoter fused to the UDPG-PPase gene. The details of vector map is showed in Fig. 3.

***E. coli* Expression vector**

An expression vector was constructed to raise antibodies for subsequent analysis of transformed plants. The expression vector was based on the fusion of the UDPG-PPase reading frame to a maltose binding domain(MBP). This pMAL vector provides a method for expression and purifying the UDPG-PPase protein in *E. coli* and is a commercially available vector allowing subsequent purification using a maltose column followed by cleavage to obtain the original UDPG-PPase protein.

EXAMPLE 2: Tobacco transformation and characterization of UDPG-PPase expression**Transformation**

The binary vector, pBIAX6 containing the Ax UDPG-PPase gene, was transformed into *A. tumefaciens* strain EHA 105 and this was used to infect *Nicotiana tabacum* c.v. xanthii leaf discs. More than 42 independent T₀ transformants were regenerated and individual plants were transferred from tissue culture into a growth room for production of seed. The stable transformation of the UDPG-PPase gene in the T₀ tobacco plants was first confirmed by PCR amplification with internal primers (see previous report). Further analyses were carried out by Southern Blot analysis. More than 42 independent transformed plants (T₀) have rooted and grown in both sterile MS medium and in soil. Seeds from 24 T₀ plants have been harvested and used to generating T₁ plants. T₁ plants were grown in soil following germination on kanamycin (150 mg/ml). Segregation of kanamycin resistant T₁ plants followed expected segregation patterns. The results demonstrated that the UDPG-PPase gene was successfully integrated into the tobacco genome. Activity of the expressed UDPG-PPase gene was assayed *in vivo* and *in vitro* by measurement of NADPH formation accompanying the enzyme-coupled conversion to 6-phosphogluconate through G-6-P. In order to test the activity of UDPG-PPase, tobacco leaves from the greenhouse were sampled using a cork borer and ground to a powder with PVPP/sand in liquid nitrogen. The enzyme was extracted with magnesium/glycine-glycine buffer and added into the assay buffer. The formation of NADPH was monitored at 340nm at 30°C continuously until a loss of the initial linear reaction rate occurred. The enzyme assay showed that UDPG-PPase activity was significantly higher in transgenic tobacco carrying the UDPG-PPase gene compared to control plants (Fig. 4). Note that in Figure 4 activity refers to specific activity (units/mg protein). Standard refers to pure commercial enzyme preparation.

Table 1. Summary of height growth (cm) of tobacco plants transformed with UDPG-PPase gene versus controls. All plant were regenerated from leaf discs.

Days of growth	Control n=9		Transformed n=42		% of control
	Avg.	SE	Avg.	SE	
1	9.02	0.94	9.67	0.40	107%
20	12.25	1.16	12.54	0.43	102%
34*	19.60	1.55	22.61	0.54	115%
47	28.60	2.06	29.17	0.80	102%

* Significant at $p=0.05$

Preliminary data on the height growth of the T_0 plants over a six week period is contained in Table 1.

During the exponential growth phase the transformed plants were significantly taller than the controls ($P=0.05$).

Segregation analysis of T_1 generation

Seeds from 16 T_0 plants were harvested and used for generating the T_1 generation. The germination rate of the T_0 seeds ranged from 52-93%, averaging 73%. Seeds for the T_1 plants were germinated on medium containing 100 or 150 ug/ml kanamycin and segregation of kanamycin resistant seedlings was scored. A Pearson chi-square test showed that most of the transformed tobacco plants contained the inserted genes in a single locus (and presumably in a single copy) due to a segregation ratio of approximately 3 to 1 (Table 2.). Scoring of kanamycin resistance in germinating T_1 seedlings was not straight forward. On water-agar, the germination frequency was very low, further, nontransformed controls and transformed seeds had similar germination frequencies. Conversely with the use of 1/2 strength MS medium, germination in the presence of kanamycin was high with all seeds, including the controls. Several parameters including root growth, cotyledon color, seedling vigor, seedling size, and the presence of primary leaves were assessed. Currently the only reproducible and reliable method for determination of kanamycin resistance in the seedlings is germination for three weeks on 1/2MS containing 150 ug/ml kanamycin and scoring resistance based on the presence or absence of primary leaves.

Table 2:

Segregation of kanamycin resistant T₁ tobacco seedlings based on presence (tolerant) or absence (susceptible) of primary leaves.

Seeds of T ₁ lines (ug/mg kanamycin)	Kan resistant	Kan sensitive	Kan+/Kan- ratio
6.01 (150)	39	13	3.0
6.03 (100)	35	14	2.5
6.05 (100)	35	12	2.8
6.06 (150)	26	15	1.7
6.07 (100)	35	12	2.9
6.08 (150)	29	10	2.9
6.09 (150)	38	12	3.2
6.12 (150)	35	14	2.5
6.13 (150)	36	10	3.6
6.14 (150)	34	12	2.8
6.15 (150)	29	8	3.6
6.23 (150)	30	14	2.1
6.24 (150)	30	18	1.7
6.25 (150)	34	15	2.3
6.33 (150)	33	11	3.0
6.42 (150)	38	12	3.2
Control5	0	44	0.0
Control8	2	46	0.0

Example 3 - Analysis of protein expression

Protein production and antigen purification

The protein expression vector pMALAX was used to overproduce the UDPG-PPase-maltose binding protein (MBP) fusion in *E. coli* after induction with isopropylthiogalactoside (IPTG). A crude protein extract was obtained with guanidine and urea buffers. Purification of the UDPG-PPase-MBP fusion protein was done by affinity chromatography using an amylose resin affinity column, with elution of the purified fusion protein from the column with 10 mM maltose. This purified fusion protein was confirmed to be a pure fraction based on SDS-PAGE and was used for antibody production in rabbits.

Antibody production

The anti-UDPG-PPase antibody was produced by Enz-Probe Biotechnology, Burnaby, B.C. after immunization of rabbits with the purified UDPG-PPase-MBP fusion protein. A UDPG-PPase specific antibody was prepared from the immunized rabbit serum by affinity purification in the presence of excess maltose binding protein (to displace antibodies which react to this portion of the fused protein). The purified antibody was used to detect the expression of proteins in Western Blotting experiments.

Protein analysis in transgenic tobacco

The protein hybridization was carried out according to Sambrook et al. (1989)⁹ with the affinity purified antibody used at a 1:500 dilution. Antibody raised against the purified protein cross-reacted on western blots with the extracted UDPG-PPase protein from both bacteria and transformed plants. Western blots showed that the antibody bound to peptides of 30 KDa and 90 kDa, corresponding to the UDPG-PPase peptide with and without the MBP fusion protein respectively. Removal of the MBP portion of the fusion protein was done by digestion with factor Xa in a modified incubation buffer. Expression of the UDPG-PPase gene in transgenic tobacco plants was inferred by the recognition of the anti-UDPG-PPase antibody to a 30 KDa peptide in the transgenic plants. Antibody binding to a peptide of similar molecular mass has never been detected in non-transformed plants.

Although not evident in Figure 6 (the Western Blot of UDPG-PPase protein with the anti-UDPG-PPase antibody), there is cross-reaction of the anti-UDPG-PPase antibody with several other bands in the protein profile of tobacco. Despite numerous experiments to further purify the antibody and increase the specificity of this antibody, the background still persists. In fact, definitive detection of the UDPG-PPase protein from individual transformed plants has been difficult because of this background. However, cleavage of the fusion protein with factor Xa (as mentioned above) provides a method to obtain higher affinity antibody to UDPG-PPase protein.

Example 4 - Cellulose analysis of transgenic tobacco.

Cellulose is one of the most important polysaccharides in tobacco and its production is directly linked to UDPG-PPase. Cellulose analysis of T_0 plants were done with both whole plants and stems from mature flowering plants. Approximately 20g (f.w.) of plant tissue was extracted with azeotropic ethanol-benzene (1:2 w/w) in a Soxhlet apparatus. After extraction, the solution was dried for soluble material analysis. The plant tissue was then ground and thoroughly mixed to make a homogenous sample. One gram of this sample was delignified with sodium chlorite in weak acetic acid and the lignin was washed away by gradual filtration. The entire polysaccharide fraction of the sample was used to determine holocellulose. Removal of hemicellulose was performed by treatment with 24% potassium hydroxide and the pure form of alpha-cellulose was recovered as a white product from filtration through a sintered crucible.

Total biomass and cellulose analysis of five control and five T_0 transformed (treated) plants showed that the transgenic plants containing the UDPG-PPase gene had significantly higher dry weight, solute content, and most importantly a-cellulose content (Figure 7). No significant differences in holocellulose were detected.

EXAMPLE 5: Protein expression and antibody production

The anti-UDPG-PPase rabbit serum was collected from rabbits after immunization with the UDPG-PPase protein produced in *E. coli*. The total antibody was assayed with enzyme-labeled protein. Antibody activity against the purified protein was detected in serum by ELISA at a titre of 32,000; a working ELISA dilution of 1/500 was used. A UDPG-PPase specific antibody, was then prepared by affinity purification in the presence of excess maltose binding domain protein to displace antibodies which react the MBP portion of the fused protein. The titre of anti-UDPG-PPase sera with affinity purification is shown in Figure 5. Note that Fractions 2 & 3 obtained by elution from an affinity column. The titre dilution is 1/1, 1/500, 1/2000, 1/8000, 1/32000, 1/128000, 1/512000, 1/2048000 contrasted with pre-immune serum (titre series 2 to 9 respectively.) The purified antibody has identified a band on a Western Blot of the same molecular weight as the UDPG-PPase protein.

Example 6: Stable transformation of spruce with 4CL-UDPG.**TRANSFORMATION OF UDPG-PPase GENE INTO SPRUCE.**

Using biolistics, transformation of spruce somatic embryos with both pBI4CLAX and pUC4CLGUS has been initiated. Over 1,500 interior spruce and 200 Sitka spruce somatic embryos have been bombarded with these constructs. The interior spruce embryos are from four different genotypes, and several different developmental stages. Following particle bombardment, the embryos were allowed to recover two weeks prior to placement on selective medium containing 5mg/ml kanamycin. Embryos were transferred every three weeks onto fresh kanamycin medium for three transfers and then placed on kanamycin-free medium

for an additional three weeks. Embryos were assessed at each transfer for the formation of callus resembling embryogenic callus characterized by clear, glassy, projections consisting of elongated cells subtended with dense head cells resembling a somatic embryo. To date, up to 4% of the embryos bombarded with pUC4CLGUS and pBI4CLAX have formed embryogenic callus on kanamycin containing medium.

Histochemical screening with x-gluc to detect GUS activity of embryogenic calli derived from embryos bombarded with the pUC4CLGUS construct has identified 22 interior spruce and one Sitka spruce transformed embryogenic lines. The GUS staining of these lines is surprisingly strong. Over 20 embryogenic lines derived from embryos bombarded with pBI4CLAX grew on kanamycin containing medium.

Example 7: Evidence of increased tolerance of drought/nutrient stress.

Four different T₁ CaMV 35S-UDPG-PPase transformed tobacco families and two control families were grown in a growth room, and not fertilized and watered only when needed for the initial 40 days. During this time, the control plants ceased growth and turned yellow, while plants from all four transformed families continued to grow, stayed green and appeared normal. After this 40 day stress period, the plants were fertilized and watered on a regular regime. Both the control plants and the transformed plants grew with the return of the watering regime and there was no noticeable difference in incremental growth between the two sets of plants. However, the control plants did never recovered fully from the stress period as they remained shorter and had less biomass than the transformed plants throughout their life. These data indicate that:

- a) The engineering of plants for the over-expression of a UDPG-PPase gene can confer increased resistance to stress.
- b) This increased resistance is due to increased vigor and not due to death of the control plants as the control plants recovered with the onset of watering.
- c) The effects of increased vigor during stress are manifested throughout the life of the plant

Growth under stress conditions

With nutrient and water stress, CaMV 35S-UDPG-PPase four different transformed families remained green and continued to grow, while the two non-transformed control families turned yellow and had greatly reduced growth (little internode elongation). These differences became noticeable approximately four weeks after establishment in soil. (Figure 8, 9) Following the first harvest (at 40 days), a regular watering and fertilizing regime was resumed and the non-transformed control plants rapidly recovered as evidenced by greening and internode elongation. In addition, after the resumption of watering there was no difference in incremental growth between the transformed and control plants. Despite this recovery, the non-transformed control plants remained significantly smaller than the transgenics throughout the experiment (Fig. 9).

Biomass measurements of stressed plants

Although a decrease in biomass was observed during the stress period in the non-transformed controls, the control plants rapidly increased in biomass with the resumption of watering and by the third harvest, the non-transformed plants had similar biomass accumulation to one of the transformed families (Fig. 10). The other three transformed lines however continued to have significantly greater biomass accumulation than the controls. The increased biomass was evident in all parts of the plant (roots, stems, and leaves) (Figure 11).

UDPG-PPase activity

There was no significant difference in the initial rate UDPG-PPase enzyme activity, however enzyme activity in the control lines peaked after 2 1/2 hours while activity continued in the transformed lines for over 4 hours (Figure 12). Thus, the transformed lines had higher UDPG-PPase activity as measure by the maximum rate of absorbance. The lack of a difference in the initial rate could be due to the fact that enzyme activity was only measured at the final harvest, 40 days after the stress had been removed, and as shown in Figure 9, incremental growth rates were the same between the control and the transformed lines at this time. It is also possible that the bacterial enzyme is more stable, yet is being masked by the plant enzyme during the initial period in this assay.

Photosynthesis and CO₂ measurements

No significant differences in photosynthesis or respiration were detected between the transformed plants and the non-transformed controls either during stress or after the removal of the stress. As expected however, during the period of nutrient and water stress photosynthetic rates were reduced relative to the later (data not shown).

Example 8: Confirmation of increased stem biomass by targeting the over-expression of UDPG-PPase to the xylem.

Using a parsley 4 coumerate:coenzyme A ligase (4CL) promoter to target the over-expression of UDPG-PPase to the xylem/vascular system, increased biomass was observed specifically in the stem. This is in contrast to the over-expression of UDPG-PPase controlled by CaMV 35S where the increase in biomass was due to increased biomass in the roots, leaves and stems. This clearly demonstrates the ability to specifically manipulate the expression of transgenes to target increases in biomass and cellulose to the stems.

Increased height of 4CL-UDPG-PPase transformed tobacco

Significant increases in height growth were observed in T₀ tobacco transformed with a 4CL-UDPG-PPase construct as shown on Figure 13. These increases were not observed until the plants started rapid growth and were maintained throughout the growth of the plant.

Increased biomass of 4CL-UDPG-PPase transformed tobacco

Significant increases in biomass were measured in T₀ tobacco transformed with a 4CL-UDPG-PPase construct. In contrast to tobacco transformed with a 35S-UDPG-PPase construct where increased biomass was observed in the roots, leaves and stem, increases in biomass in tobacco transformed with a 4CL-UDPG-PPase construct was restricted to the stems. Figure 14 demonstrates the efficacy of xylem directed targeting of the expression of the UDPG-PPase gene.

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We Claim:

1. A process of increasing plant growth and yield which comprises introducing into a plant a DNA sequence encoding a product which modifies, in the plant, the level of cellulose precursors.
2. The process of claim 1 wherein the product is selected from the group consisting of enzymes, proteins and ribonucleic acid.
3. The process of claim 1 wherein the product is a carbohydrate modifying enzyme selected from the group consisting of uridine diphosphate glucose pyrophosphorylase (UDPG-PPase), sucrose synthetase and cellulose synthetase.
4. The process of claim 1 wherein the product regulates the expression of a cellulose-dependent enzyme.
5. The process of claim 1 wherein the precursor is uridine diphosphate glucose (UDP-glucose).
6. The process of claim 1 wherein the plant is a tree.
7. The process of claim 1 wherein the DNA sequence comprises the UDPG-PPase gene derived from a bacterium.
8. The process of claim 1 wherein the DNA sequence comprises the UDPG-PPase gene derived from *Acetobacter xylinum*.
9. A process of modifying the production of cellulose in a plant which comprises introducing into and expressing in said plant a DNA sequence encoding a product which modifies the level of cellulose precursors in the plant.
10. The process of claim 9 wherein the product is selected from the group consisting of enzymes, proteins and ribonucleic acid.
11. The process of claim 9 wherein the product is a carbohydrate modifying enzyme selected from the

group consisting of UDPG-PPase, sucrose synthetase and cellulose synthetase.

12. The process of claim 9 wherein the precursor is UDP-glucose.

13. The process of claim 9 wherein the plant is a tree.

14. A process of modifying the production of cellulose in a plant which comprises introducing into and expressing in said plant a DNA sequence comprising the UDPG-PPase gene operably linked to an expression effecting DNA sequence and flanked by translational start and stop signals.

15. The process of claim 14 wherein the expression effecting DNA sequence is a promoter directing expression of the UDPG-PPase gene primarily in cellulose-producing cells of the plant.

16. The process of claim 14 wherein the expression effecting DNA sequence is a promoter selected from the group consisting of one or more of CaMV 35S and 4CL from parsley.

17. The process of claim 14 wherein the plant is a tree.

18. A plant having modified cellulose producing activity as a result of introducing into said plant or parent of said plant a DNA sequence coding for a product which modifies the level of cellulose precursors in the plant.

19. The plant of claim 18 wherein the product is an enzyme.

20. The plant of claim 18 wherein the product is a carbohydrate modifying enzyme selected from the group consisting of UDPG-PPase, sucrose synthetase and cellulose synthetase.

21. The plant of claim 18 wherein the product regulates the expression of a cellulose-dependent enzyme.

22. The plant of claim 18 wherein the precursor is UDP-glucose.

23. The plant of claim 18 being a tree.

24. The plant of claim 18 wherein the DNA sequence comprises the UDPG-PPase gene derived from a bacterium.
25. A process of increasing the resistance of a plant to stress which comprises introducing into the plant a DNA sequence encoding a product which modifies, in the plant, the level of cellulose precursors.
26. The process of claim 25 wherein the product is a carbohydrate modifying enzyme selected from the group consisting of uridine diphosphate glucose pyrophosphorylase (UDPG-PPase), sucrose synthetase and cellulose synthetase.
27. A DNA expression vector comprising a DNA sequence encoding a bacterial protein which modifies the level of cellulose precursors, said DNA sequence being operably linked to an expression effecting DNA sequence and flanked by translational start and stop sequences.
28. A genetically modified seed product comprising a DNA sequence encoding a product capable of modifying the level of cellulose precursors in the plant resulting from the seed product.

FIGURE 1

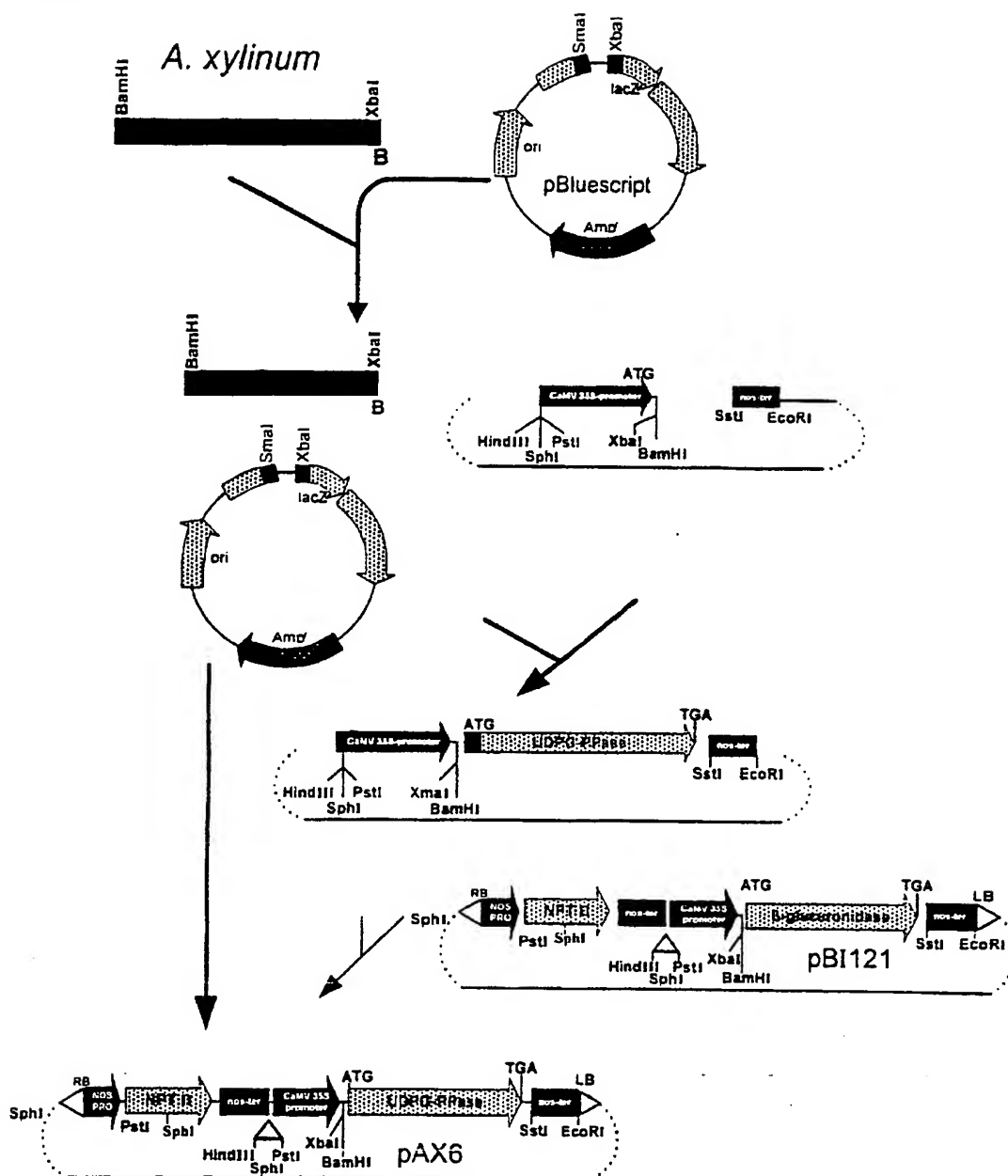


FIGURE 2

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      .      V I K P L K K A V L P V A G L G T R F L P A T K C 25
A. xylinum  ccacgcatctcgaggtaaatattcGTGattaagcccttcaaaaaagctgattgcccgggtgcccggccttggaaacacgctttctgcccgcacccaagtgcg 100
pAX6 binary vector  cggtraccATGGTCAAGCCCTTAAAAAAGCCCTATTGCCGCTTCCGSCCTTGGAAACATGCTTCTGCCCCGCCACCAAGTGGG 100
      M V K P L K K A V L P V A G L G T R F L P A T K C 33

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A. xylinum  tgcaccaaggaaatgctgaccgcttctgaccgtccgctgacccagtatgcqattgacgaggccacgcgaagccgggacgaggaattctgctcgtttccag 200
pAX6        TGCCCAAGGAAATGCTGACCGTCTTTGACCGTCCGCTGATCCAGTATGCGATTGACGAGGCACCGGAAGCCGGGCTCGAGGAATTCTGCTCCTTTCCAG 200
      V P K E M L T V V D R P L : Q Y A I D E A R E A G V E E F C L V S S 67

      R G K D S L I D Y F D I S Y E L E D T L K A R K K T S A L K A L E 92
A. xylinum  cccggggcaaggattccctgacgattatttcacacatttctcaccgaactcgaadacacgctgaaggcccgcaagaagacatcgccactgaaggccctcgaa 300
pAX6        CCGGGGCAAGGATTCCCTGACGATTATTCGACATTTCATACGAACCTCGAAGACACGCTGAAGGCCCGCAAGAAGACATCGGCCTGAAGGCCCTCGAA 300
      R G K D S L I D Y F D I S Y E L E D T L K A R K K T S A L K A L E 100

      A T R V I P G T M L S
A. xylinum  gcaaccccgctcatcccgggcaccatgctgtccg
pAX6        GCAACCCGCTCATCCCGGCACCATGTTGTCCG
      A T R V I P G T M L S

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FIGURE 3

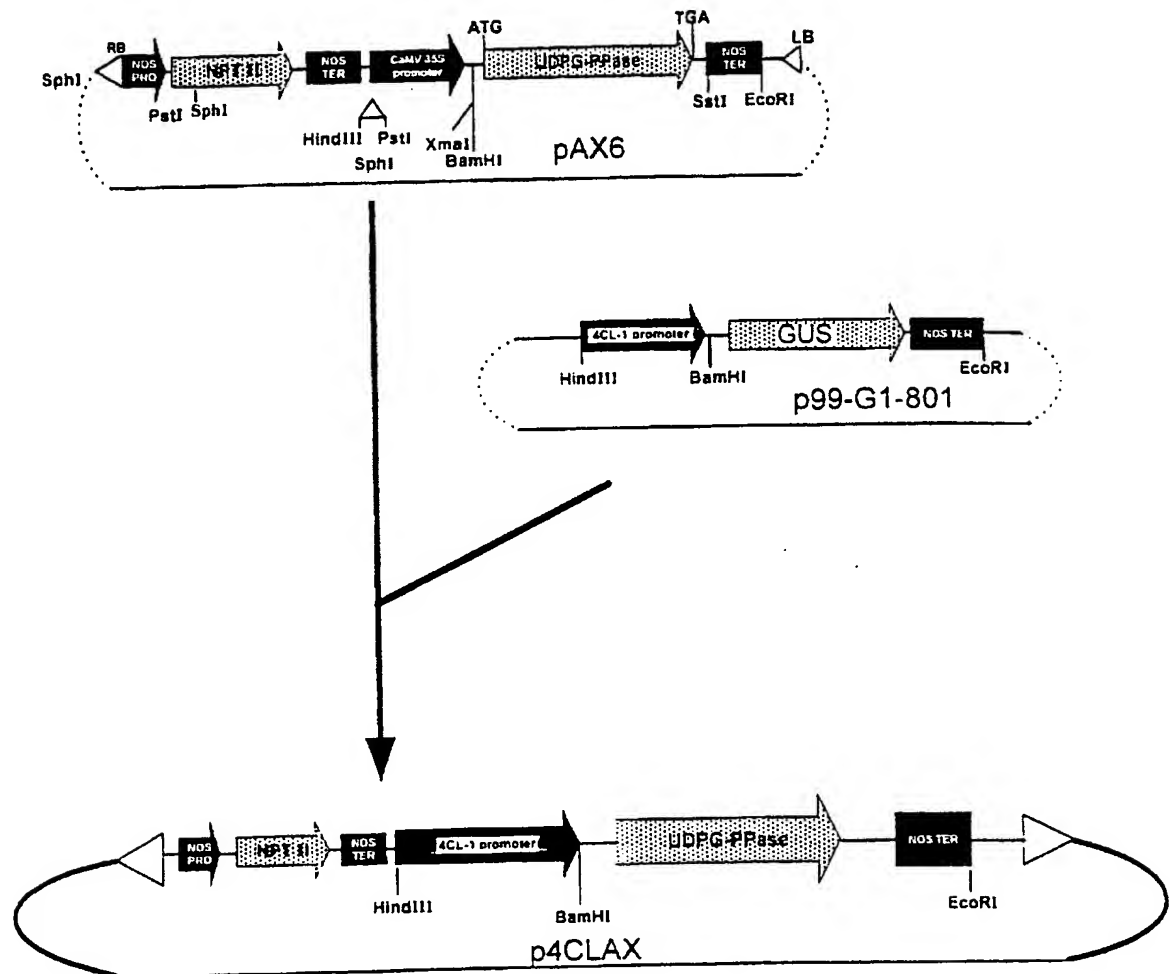


FIGURE. 4

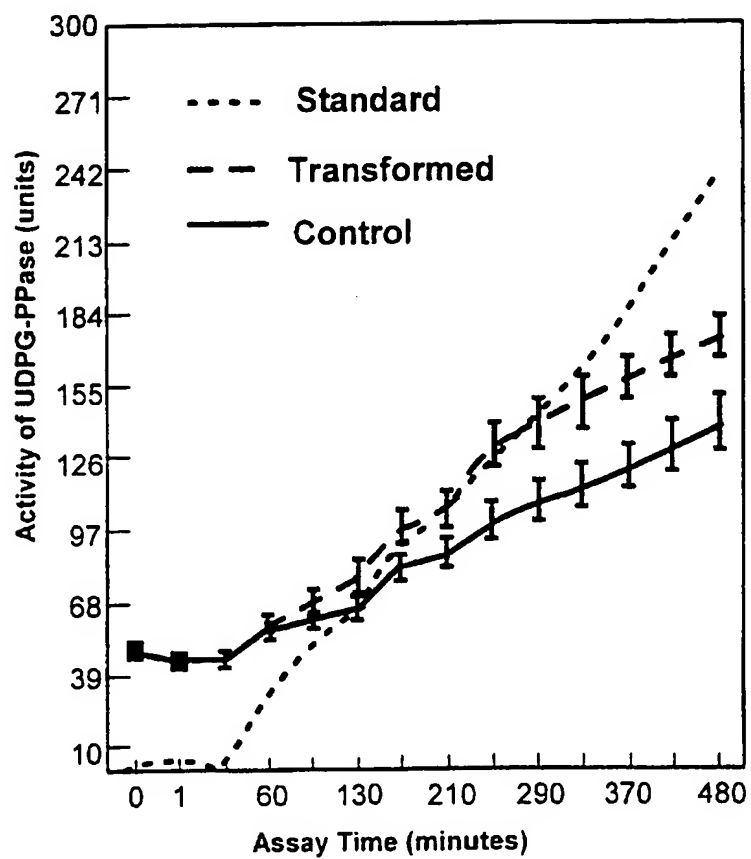
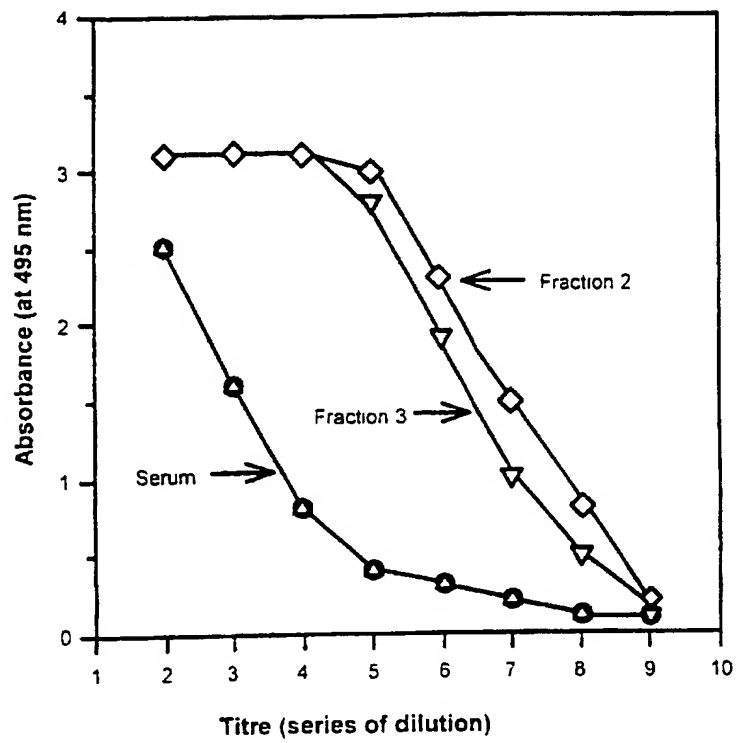


FIGURE. 5



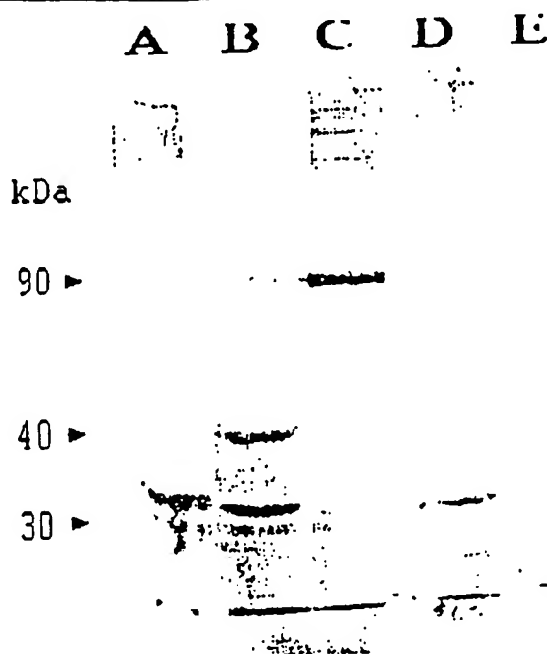


Figure 6

Proteins extracted from: Lane A, *A. xylinum* showing the UDPG-PPase protein of approximately 30 KDa; lane B, *E. coli* expressing pMALAX digested with factor Xa showing 40 and 30 KDa bands corresponding to the MBP and UDPG-PPase proteins respectively; lane C, *E. coli* expressing pMALAX without digestion showing a 90 KDa band corresponding to the UDPG-PPase-MBP fusion protein; lane D, transgenic T₀ tobacco plants (protein pooled from 5 independent transformants) showing antibody recognition of a 30 KDa peptide corresponding in relative molecular weight to the *A. xylinum* UDPG-PPase protein; and lane E, control non-transformed tobacco plant showing no antibody binding to peptides in the 30 KDa size region.

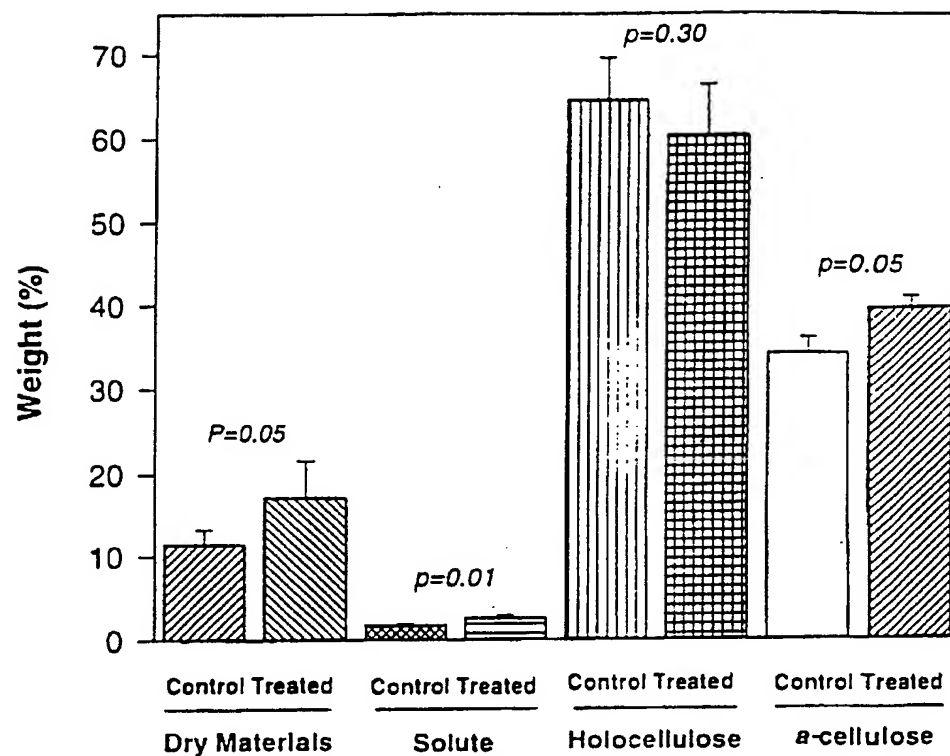


Figure 7

FIGURE 8



Figure 9

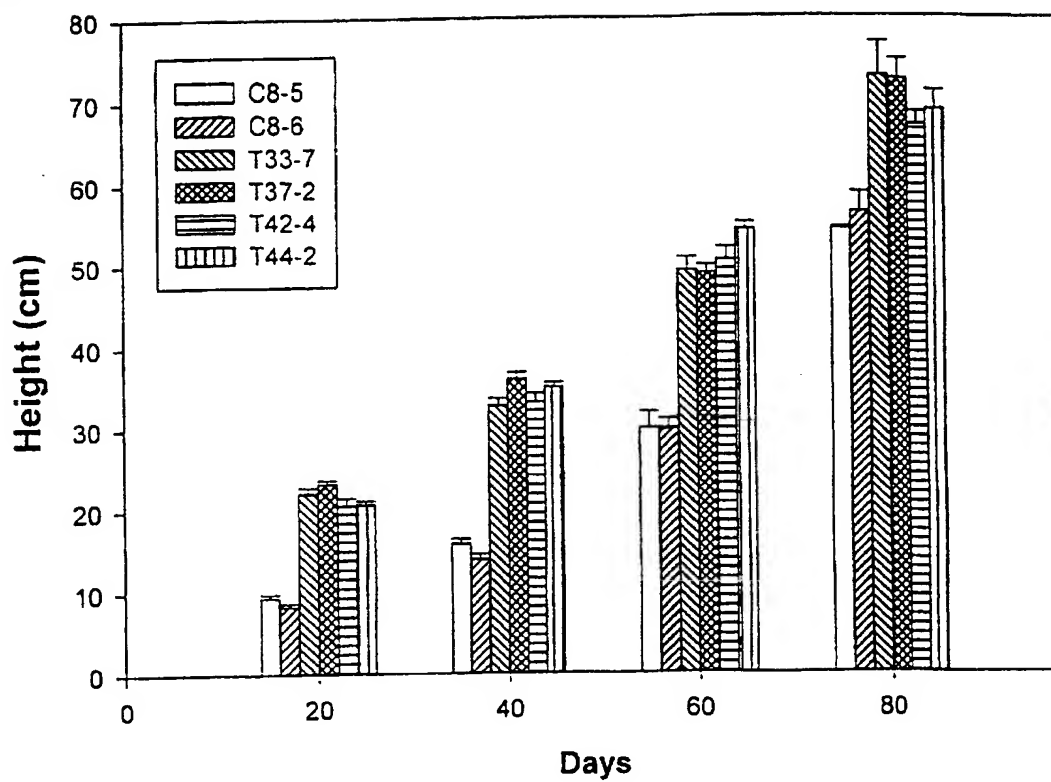


Figure 10

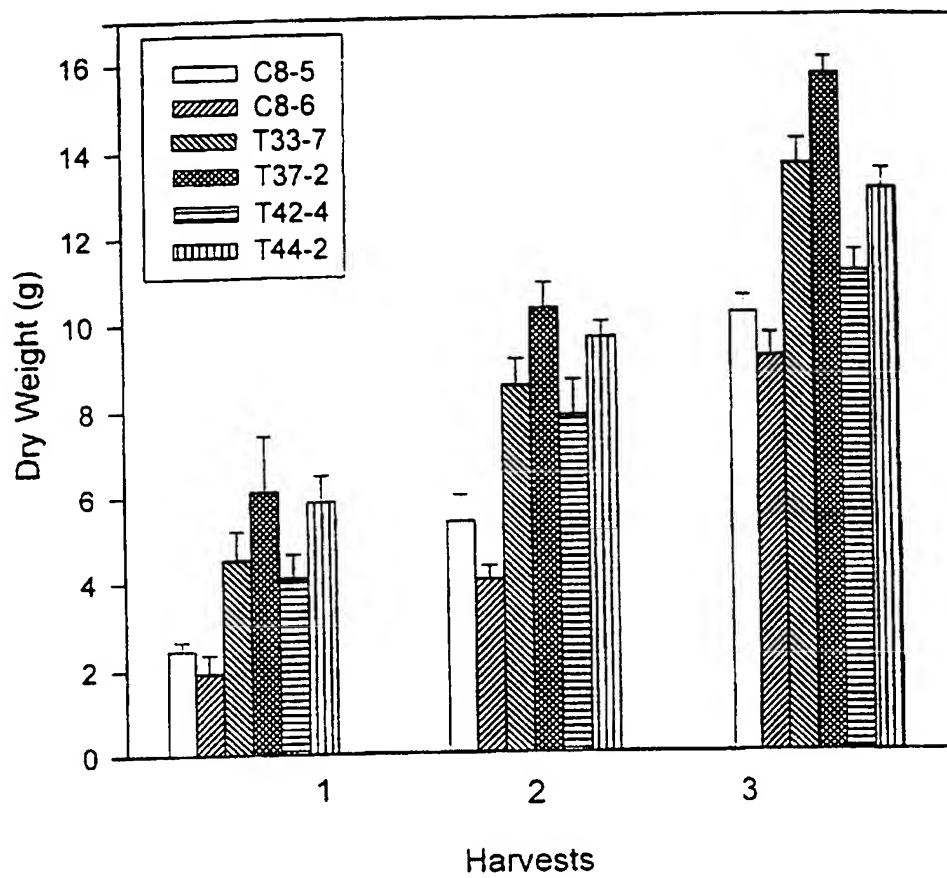


Figure 11

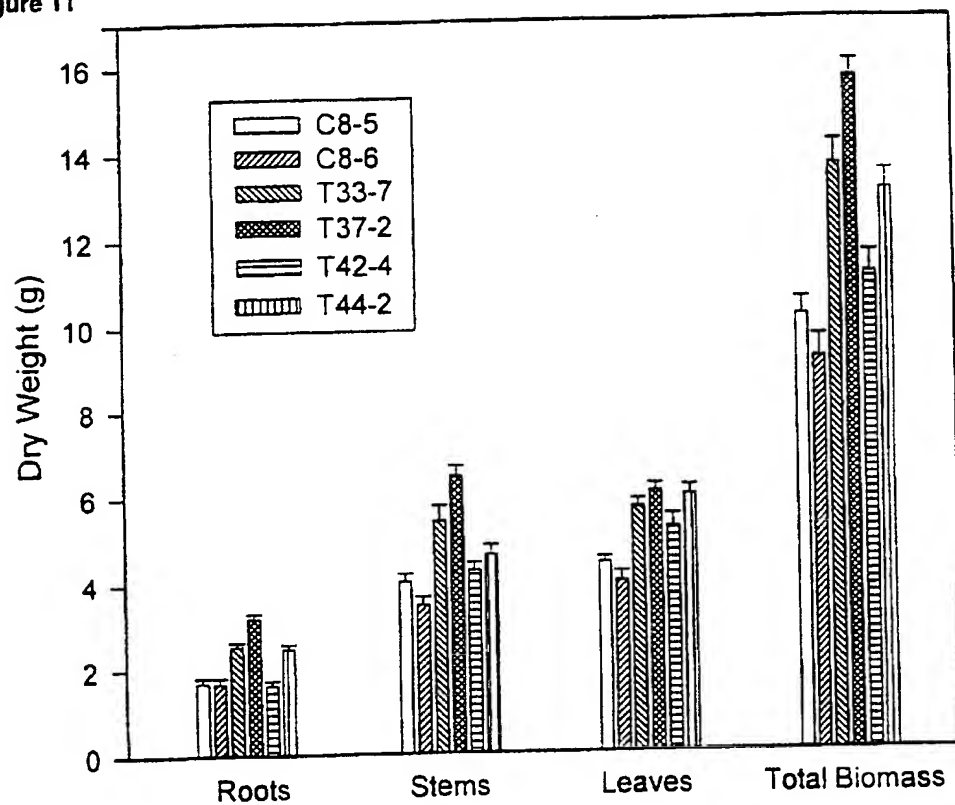


Figure 12

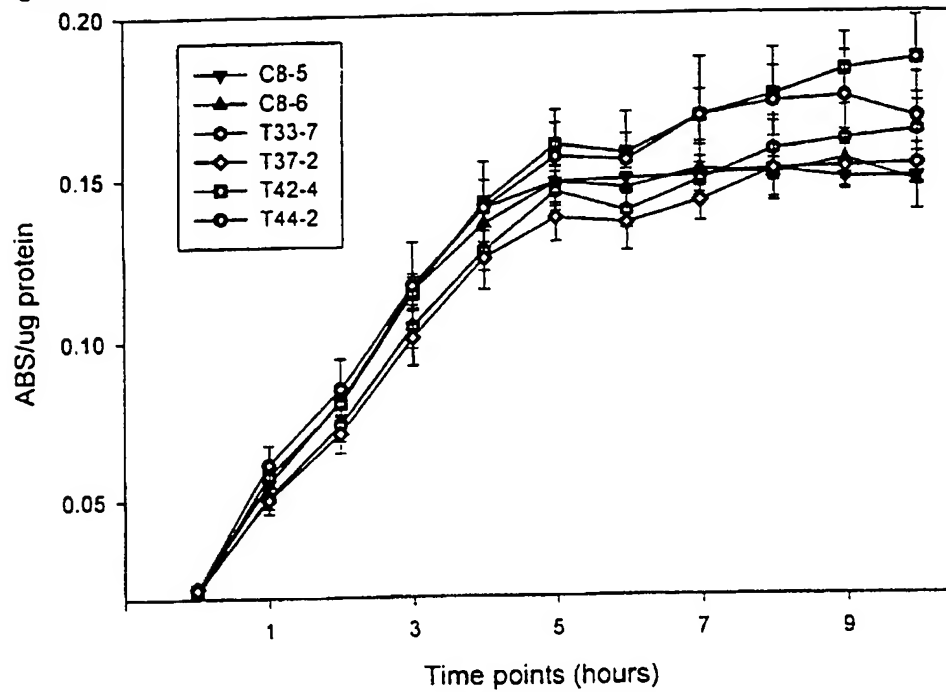


Figure 13

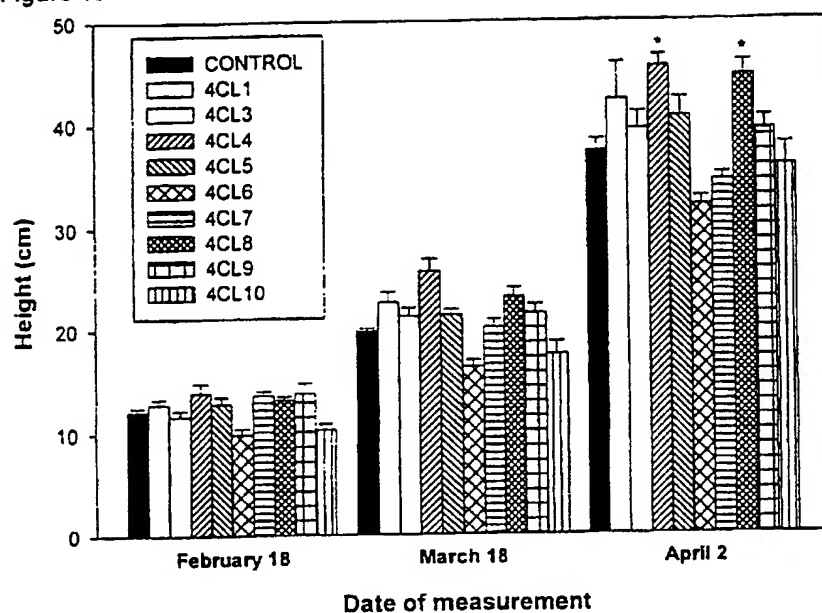
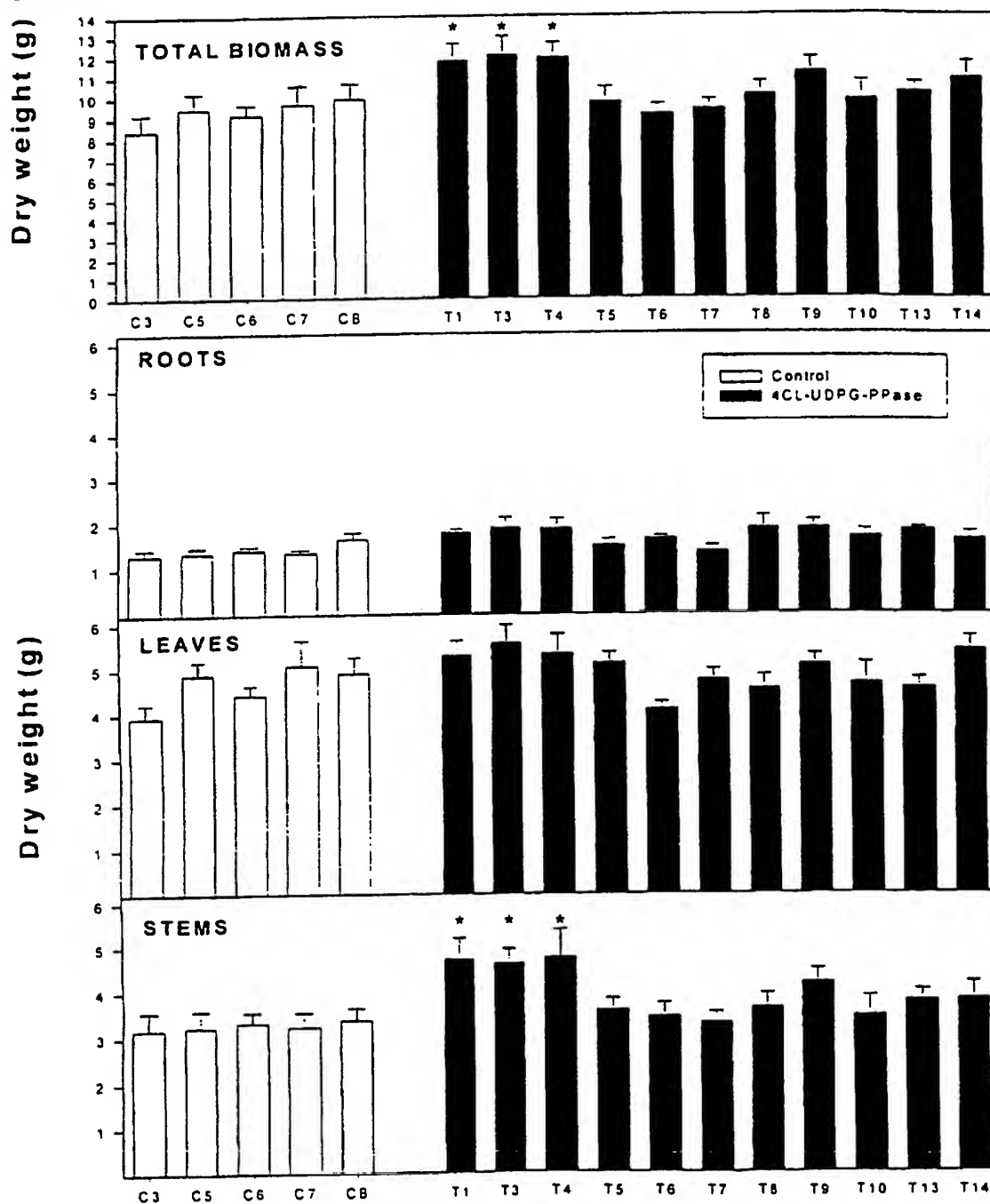


Figure 14



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